REMARKS

Applicants have carefully considered this Application in connection with the Examiner's Action, and respectfully request reconsideration of this Application in view of the above Amendment and the following remarks.

Applicants have amended the Specification at .

Applicants have amended Claims 1, 11, 76, and 86. Applicants have cancelled Claims 2, 3, 77, and 78. Applicants have added new Claims 137 and 138.

Claims 1 and 76 have been amended to clarify that the vector is delivered into either the diploid or the muscle cells of the female animal. Support for this amendment can be found in the Specification at paragraphs 15 and 20, as well as in the original disclosure of cancelled Claims 2, 3, 77, and 78.

Claims 11 and 86 have been amended to clarify that the vector can be a plasmid or a viral vector. Support for this amendment can be found in the Specification at paragraphs 15 and 20. Applicants have also added new Claims 137 and 138, which provide that the vector can be formulated with a liposome, a cationic lipid, or a combination thereof. Support for this amendment can be found in the Specification at paragraphs 15 and 20.

Pending in the application are Claims 1 – 13, 76 – 88, and 137 – 138.

1. **Notice to Comply with Requirements for Patent Applications Containing Nucleotide Sequence And/Or Amino Acid Sequence Disclosures**

The Examiner has held that the amino acid sequences in Figure 1 of the application do not comply with the Requirements for Patent Applications Containing Nucleotide Sequence And/Or Amino Acid Sequence Disclosures. Specifically, the Examiner held that the application fails to comply with C.F.R. 1.821(d).

In response, Applicants have amended the brief description of figures to include sequence identifiers. More specifically, paragraph [0025] has been amended to include the phrase "SEQ ID NO: 1" to identify the porcine wild type (1-40)OH amino acid sequence, and the phrase "SEQ ID NO: 8" to identify the analog HV-GHRH in Figure 1A.

Applicants believe that the amended paragraph [0025] places the application in compliance with the sequence rule C.F.R. 1.821(d). There is no need to submit an amended substitute computer readable or paper copy of the "sequence listing." The reason is that both "SEQ ID NO: 1" and "SEQ ID NO: 8" were already included in the most recent sequence listing submitted to the Patent Office on July 12, 2002, under the Statement Under 37 C.F.R. § 1.821 (F).

2. Rejections Under 35 U.S.C. §112

A. Enablement

Claims 1 – 13 and 76 – 88 stand rejected under 35 U.S.C. §112, first paragraph, for being non-enabled. The Examiner asserts that the claimed invention encompasses *in utero* gene therapy, which is unpredictable, and that the specification does not provide sufficient guidance.

Applicants respectfully assert that the claims as amended are fully enabled and that the effectiveness and utility of the claimed methods are established in the Specification and are not at issue. As noted above, Applicants have amended the claims to clarify that the vector is delivered into either diploid or muscle cells of the female animal. Thus, the claims as amended do not pertain to "*in utero* gene therapy" because the vector must be delivered into diploid or muscle cells of the female animal.

"I*n utero*" gene therapy," as described by gene therapy pioneer W. French Anderson, is : **"to transfer a therapeutic gene into a child with a genetic."** *See*, "NIH Committee Debates Future of *In Utero* Gene Therapy," *HSC Weekly*, Volume 4, Issue 26, September 25, 1998, page 1, 6th paragraph, a copy of which is attached as Exhibit 1. In a review article by E. D. Zanjani and W. French Anderson, the authors described "*in utero* gene therapy ("IUGT")" as "**gene transfer in the**

fetus . . . ." *See* "Prospects for In Utero Human Gene Therapy," *Science*, Vol. 285, page 2084, first column, 24 September 1999, a copy of which is attached as Exhibit 2. Thus, *"in utero* gene therapy" **DOES NOT** encompass the broad definition given by the Examiner, which is: "**any nucleic acid linked to any promoter and any 3' UTR is introduced into any animal at any time (before, after or during pregnancy) by any method/route so as to increase growth of the offspring.**" *See* Office Action, page 2, last four lines. In fact, under the broad definition given in the Office Action, a mother's better nutrition or consumption of food prior to, after, or during pregnancy, which better nutrition or consumption of food positively correlates with higher birth weights and increased growth of offspring, would be a form of "*in utero* fetal gene therapy." The reason is that food contains highly variable quantities of genomic material (i.e. any nucleic acid linked to any promoter and any 3'UTR) and is introduced into the mother at any time, before, after, or during pregnancy, by any method or route. This broad interpretation of "*in utero* fetal gene therapy," of course, cannot be correct.

The current claims, as amended, **do not** encompass "*in utero* gene therapy." Plasmids are not being delivered to the fetus. Rather, plasmids are delivered to either diploid or muscles of the female animal whose expression of the delivered nucleic material improves the growth and health of the offspring.

The Examiner asserts that Khan et al., *Endocrinology* (2002) and Khan et al., *Amer. J. Physiol.* (2003), which were both published **after** the filing date of the current application, demonstrate that "[b]oth the pigs and the rat offspring showed enhanced growth and muscle hypertrophy." *See* Office Action, page 3, last 2 lines.

Applicants respectfully disagree. As clearly shown in Khan et al., *Endocrinology* (2002), the rats had increased body weights to 10 weeks of age, but by 24 weeks of age the difference was no longer significant (*see* Results, page 3562). Also, as far as the muscle hypertrophy was concerned, only female offspring had muscle hypertrophy maintained for 24 weeks, but not males which have higher testosterone levels. The data point towards a faster maturation of the rat offspring that stays within PHYSIOLOGICAL stimulation of the GHRH axis and not to an unpredictable "*in utero* gene

therapy." As shown in Khan et al., *Amer. J. Physiol.* (2003), offspring of treated sows are heavier from birth to slaughter, within physiological variability. There is no description of muscle hypertrophy in the pigs.

The Examiner also asserts that "there is no way of controlling the expression of the gene and therefore delivery of the protein to the fetus could not be controlled and as seen this would lead to muscle hypertrophy in the offspring which may not be desirable in a human subject." *See* Office Action, page 4, lines 4 – 8.

Again, Applicants respectfully disagree. First, there is no data presented in the Specification which indicates that this therapy would have the effect of muscle hypertrophy on a human. Second, Applicants respectfully point out that in Khan et al., *Amer. J. Physiol.* (2003), a study of the circulation of the injected plasmid was undertaken. As clearly shown, plasmid does not circulate from the injection point, and all samples from sow's milk, colostrums, **amniotic fluid**, and **placenta**, and from offspring liver were **negative** for the presence of plasmid. Thus, the study indicates that **no plasmid crosses to the fetus**, so the therapy **is not, and cannot be, an "*in utero* fetal gene therapy**." Rather, although not wanting to be bound by the postulation, Applicants postulated that physiologic feed-back mechanisms in the mother control the levels of GHRH that could arrive to the fetus, making precise control of plasmid transcription unnecessary.

The Examiner has also cited Stribley et al., Fertility and Sterility (2002) ("Stribley"), to demonstrate the "significant problems" and "challenges" of gene therapy and to assert that it was not routine in 2002. In particular, the Examiner cites Table 1 of Stribley. However, Applicants respectfully point the Examiner's attention to the Specification's evidence of the utility and effectiveness of the claimed methods. Applicants have addressed the "challenges" of Stribley's Table 1. An appropriate gene was chosen: GHRH. An appropriate method of delivery was used: intramuscular injection followed by electroporation. Transfection was achieved for the target period of time, which was 30-50 days. In addition, the GHRH axis is naturally regulated by feed-back mechanisms at multiple levels. There were no adverse effects on the treated animals and their offspring. In all treated animals in the studies described in the Specification, published studies, and

hundreds of other animals enrolled in different studies, no adverse effects linked to the plasmid were observed. Furthermore, the results with this therapy are better than conventional therapies. The offspring from treated animals are slightly bigger at birth, thus more resistant to disease or other challenges. GHRH positively impacts the offspring immune function, and thus these animals are healthier. No antibiotic treatments or growth promotants are necessary. Lastly, feed efficiency is improved, so these animals are generating less manure, with positive impact on the environment.

In addition, the claimed technique does not require viral vectors, such as adenoviruses, retroviruses, or adeno-associated viruses which are discussed at length in Stribley. Furthermore, Stribley does not entirely rule out the feasibility of direct DNA delivery. Stribley states at page 651, second paragraph that for direct DNA delivery, "[s]o far, the tissue exhibiting the highest success is muscle." And despite the fact that the vectors, target tissue, conditions, etc., are not known for many situations, Stribley quotes on page 652 that under "clinical applications of gene therapy," children with a severe combined immunodeficiency have recently been successfully cured using a gene therapy technique. Stribley also clearly establishes that "*in utero* fetal gene therapy" means the administration of a gene therapy vector directly to the fetus and not to the mother of the fetus. See Stribley at page 652, column 2.

The Examiner has also cited Anderson, Nature (1998) as indicating that "the efficiency of gene transfer and expression in human patents is, however, still disappointingly low." *See* Office Action, page 5, first full paragraph. Anderson underlines a problem that existed when the article was published, that of low efficacy of gene transfer that cannot ensure physiologic levels of a circulating protein, enzyme or hormone capable of restoring normal levels in a human patient. Applicants respectfully assert that the Examiner has ignored the evidence regarding the effectiveness of the current therapy which are included in the Specification. The Examples demonstrate that the claimed methods overcome this very problem, by using direct plasmid injection intramuscularly followed by electroporation. In the cited article, Dr. Anderson underlines that fact that "although viral systems are potentially very efficient, two factors suggest that non-viral (i.e. plasmid DNA) gene delivery systems will be the preferred choice in the future: safety and ease of manufacturing." *See* page 28, in the "non-viral vectors" paragraph. Furthermore, the levels of circulating GHRH necessary to

obtain physiological stimulation of the GHRH axis, and beneficial effects on the offspring are very low when compared with other peptides. Thus, using the methods presented in the Specification, stable levels of GHRH at a physiological concentration are ensured.

Romano et al, Stem Cells (2000) ("Romano") is also cited by the Examiner as discussing the "unpredictability with the issues of vectors, promoter, route of administration." *See* Office Action, page 5, last three lines. In Romano, however, the authors emphasize different aspects of gene therapy using viral vectors targeted to infectious diseases, in particular HIV/AIDS. Furthermore, on page 20, second column, second paragraph, the author clearly defines "*in utero* gene therapy" as "another line of intervention" that was recently proposed. "These clinical trials envisage the treatment and/or prevention of certain inherited genetic disorders, which may have catastrophic outcomes in children." However, as noted above, the claimed methods of the present application do not pertain to "*in utero* gene therapy," and in particular they do not pertain to the treatment or prevention of certain inherited genetic disorders.

In light of the Amendment and the remarks above, Applicants' claimed methods **DO NOT pertain to "*in utero* gene therapy."** Applicant's **claimed methods are also supported by sufficient evidence and actual working examples in the Specification to show their effectiveness**. Thus, despite the Examiner's concerns (based on theories) and the number of cited references (none of which is **identical** to the invention of the present application), for which Applicants are grateful, the **Specification and the actual working examples therein demonstrate that the claimed methods do work as claimed and are enabled**.

For these reasons, Claims 1 – 13 and 76 – 88 are patentable under 35 U.S.C. §112, first paragraph.

B. Indefiniteness

Claims 11 and 86 stand rejected under 35 U.S.C. §112, second paragraph, as being indefinite. The Examiner asserts that it is unclear as to how a vector could comprise a liposome or lipid.

Applicants have amended Claims 11 and 86 above to clarify that the vector can be a plasmid or a viral vector. Applicants have also added new Claims 137 and 138, which specify that the vector can be *formulated* with a liposome, a cationic lipid, or a combination thereof.

In light of the above Amendment, Applicants respectfully assert that Claims 11 and 86 are patentable under 35 U.S.C. §112, second paragraph.

3. Conclusion

Applicants respectfully submit that, in light of the foregoing Amendment and comments, Claims are in condition for allowance. A Notice of Allowance is therefore respectfully requested.

If the Examiner has any other matters which pertain to this Application, the Examiner is encouraged to contact the undersigned to resolve these matters by Examiner's Amendment where possible.

Respectfully submitted,

T. Ling Chwang
Registration No. 33,590
JACKSON WALKER L.L.P.
2435 North Central Expressway, #600
Richardson, TX 75080
Tel: (972) 744-2919
Fax: (972) 744-2909

Nov. 5, 2004
Date

EXHIBIT 1

French Anderson

NIH committee debates future of *in utero* gene therapy

by
Eva Emerson

USC gene therapy pioneer W. French Anderson is ready to explore a new frontier in genetic medicine - correcting genetic disorders before children are even born.

Anderson, professor of biochemistry and molecular biology and director of the USC Gene Therapy Laboratories, and his colleagues have submitted the first proposals to do in utero gene therapy to the Office of Recombinant DNA Activities at the National Institutes of Health.

Although this marks one of the first steps toward the approval of clinical trials, the submission of the so-called pre-protocols is simply meant to initiate discussion and is not considered part of a formal approval process.

The proposals, to be discussed at the Sept. 24 and 25 meeting of the national Recombinant DNA Advisory Committee (RAC), outline Anderson's aim to develop clinical protocols to use bio-engineered viral vectors to cure genetic diseases before birth.

The committee will discuss the scientific, ethical and public policy issues related to attempts at in utero gene therapy, with Anderson's pre-protocols taking center stage.

"This is something that's never been attempted, but the success of our large animal experiments suggests it may be an effective and relatively safe way to transfer a therapeutic gene into a child with a genetic disorder," said Anderson, who was at the NIH campus to discuss the matter this week.

"At this point, we're not asking for any kind of approval," said Anderson, who characterizes the proposals as "conservative and facts-only statements backed up with a lot of data."

"We are still two to three years away from requesting approval to use an in utero technique in a patient. We just want to open up the public discussion," Anderson said. He notes that reviews of the proposals from other scientists and ethicists have been surprisingly favorable.

"This is very different from the situation 10 years ago. When we initially put out our idea to do the first gene therapy trial in 1987, all 14 of our reviews were extremely negative. This time, all 18 reviews were supportive. Some even thanked us for putting it out there," he said.

"I want to thank Dr. Anderson and his colleagues for submitting these drafts to the RAC. I applaud the effort to solicit as much public and peer discussion at this early stage as possible," wrote Claudia Mickelson, a scientist at Massachusetts Institute of Technology and chair of the RAC, in her review. "Dr. Anderson's willingness to open the discussion on the use of gene transfer in this patient population is quite audacious," she wrote.

Anderson has developed a new generation of improved gene therapy delivery tools that he thinks will allow more efficient gene transfer. As well, it may be that doing gene therapy on a fetus could work better than on children or newborns. That is because the viral-derived vectors carrying the corrective gene can only enter dividing cells, and the cells of the rapidly developing fetus divide often.

The pre-protocols also raise the possibility that doing gene therapy in utero could increase the chance of genetically

altering sperm or egg cells, changes that would be passed on to offspring.

Although that is not the goal of the proposed therapy, that potential does bring up the issue the safety and desirability of germline engineering, an item sure-to-be debated at the meeting.

The pre-protocols deal with genetic disorders both of which have been linked to dysfunction in a single gene. Some cases of severe combined immunodeficiency (SCID) - commonly known as the bubble baby disease - are caused by a flaw in the gene that encodes for adenosine deaminase (ADA) and leaves a child without a functional immune system.

The rare, often fatal disorder caused by an ADA-deficiency was the first disease in which gene therapy was attempted, in 1990, by Anderson. In 1993, USC faculty at Childrens Hospital Los Angeles performed the first gene transfer into the blood stem cells isolated from the umbilical cord in newborn patients with this same disease.

Anderson proposes to inject a corrective ADA gene attached to an improved retroviral vector into a fetus early in the second trimester.

Also proposed is a genetic treatment for alpha-thalassemia, a very common genetic disease that, in the most severe cases, results in death in the womb. The disease results from defects in the alpha forms of hemoglobin, the oxygen-carrying molecules of the blood.

For this procedure, Anderson proposes delivering gene therapy to blood stem cells taken from the fetus. These transformed stem cells would then be re-introduced into the developing fetus, where they would help repopulate the body with gene-corrected blood cells.

Previous story
Next story
September 25, 1998 Issue Index

HSC Public Relations page
HSC Weekly index

Last modified September 28, 1998 □□

EXHIBIT 2

BIOLOGY OF REPRODUCTION 54, 682–691 (1996)

Transduction of Human Trophoblast Cells by Recombinant Adenoviruses Is Differentiation Dependent[1]

Colin D. MacCalman,[3] Emma E. Furth,[4] Akinyinka Omigbodun,[3] Karen F. Kozarsky,[5,6] Christos Coutifaris,[3] and Jerome F. Strauss III[2,3,4]

Department of Obstetrics and Gynecology,[3] Department of Pathology and Laboratory Medicine[4] Institute of Human Gene Therapy, Department of Molecular and Cellular Engineering[5] University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104 Wistar Institute,[6] Philadelphia, Pennsylvania 19104

ABSTRACT

To explore the feasibility of adenoviral (Ad)-mediated gene transfer to the human placenta, we examined the ability of two recombinant Ad vectors to transduce isolated human cytotrophoblast cells and two choriocarcinoma cell lines (BeWo and JEG-3, which have different potentials to undergo morphological differentiation in response to cAMP). Recombinant Ad efficiently transduced cytotrophoblast cells. However, there was a marked reduction in the transduction efficiency of these vectors after the terminal differentiation of the mononucleate cytotrophoblasts into multinucleate syncytial trophoblast. BeWo and JEG-3 cells were readily transduced with the recombinant Ad, but a striking reduction in transduction efficiency of the Ad vector was observed in BeWo cells following cAMP-stimulated cellular differentiation, which includes cell fusion to form syncytia. In contrast, JEG-3 cells, which are not induced to fuse in the presence of cAMP, did not show a reduced transduction efficiency when exposed to the cyclic nucleotide. Reporter gene copy number increased with Ad-mediated gene transfer into undifferentiated Bewo cells but was low in cells that had been previously exposed to cAMP. In contrast, both undifferentiated and cAMP-treated BeWo cells were capable of expressing a reporter gene when transfected with an Ad-based plasmid. Taken together, these results demonstrate that the reduction in transduction efficiency of the Ad vectors in cAMP-treated BeWo is the result of reduced infectivity rather than of a reduction in the transcription/translation efficiency of the exogenous genes. Our findings demonstrate that recombinant Ad vectors will not be useful for the transfer of genes into differentiated trophoblast cells because these cells are resistant to Ad infection. This may limit the utility of Ad-based vectors for placental gene therapy. However, we have documented that less-differentiated trophoblast cells are susceptible to Ad-mediated gene transfer. Our observations also suggest a mechanism by which differentiated human trophoblast cells resist Ad infection and prevent fetal infection by maternally derived Ad.

INTRODUCTION

The placenta plays a central role in supporting fetal growth [1–3]. It is the site of transfer of respiratory gases, nutrients, and waste products between the fetal and maternal systems; it serves as a barrier against pathogens and the maternal immune system; and it is an active endocrine organ secreting hormones, growth factors, and other bioactive substances.

The epithelial cells of the placenta are derived from the trophectoderm of the blastocyst [4, 5]. Upon implantation in the human, the mononucleate cytotrophoblast cells rapidly divide, invade the endometrium, and subsequently differentiate [6]. The morphological and functional differentiation of the cytotrophoblast stem cells ends with the formation of the multinucleated syncytial trophoblast of the chorionic villi.

In the human, failure of placentation and placental insufficiency can have severe consequences for the health of the mother and fetus. For example, abnormalities in trophoblast invasion may cause preeclampsia, a disease associated with significant maternal and fetal morbidity and mortality [7–9]. Inadequate placental function, which frequently accompanies preeclampsia, leads to intrauterine growth retardation [10]. Currently, the medical therapies available for treatment of preeclampsia and other disorders of placental function are limited and often ineffective [11]. The development of strategies for gene therapy of the placenta could provide an alternative for the therapeutic management of placental dysfunction.

Replication-deficient recombinant adenoviruses (Ad) represent a gene delivery system that could be used to transfer exogenous genes into placental cells. Recombinant Ad vectors have been used to introduce genes efficiently into a wide variety of tissues and cell types in vitro and in vivo [12–19]. In addition, Ad has low pathogenicity in humans, can accommodate relatively large segments of DNA (up to 7.5 kb), and can transfer genes into nonproliferating cells [20–22].

We used two recombinant Ad vectors containing model genes to explore the ability of Ad vectors to transfer genes into human placental cells. The first, Ad.CMV*lacZ*, is a recombinant Ad vector containing the *Escherichia coli LacZ* gene, coding for the intracellular protein β-galactosidase (β-gal) [23]. This construct was used to examine the transfer efficiency of a gene coding for an intracellular protein and,

Accepted November 14, 1995.
Received October 4, 1995.
[1]This work was supported by NIH grants HD-29946, HL-50786, HD-06274, and a grant from the W.W. Smith Charitable Trust.
[2]Correspondence: Jerome F. Strauss III, M.D., Ph.D., University of Pennsylvania, Dept. of Obstetrics & Gynecology, 778 Clinical Research Building, 415 Curie Boulevard, Philadelphia, PA 19104. FAX: (215) 573-5408.

in parallel, to identify those cells that can be modified by an exogenous gene. The second recombinant Ad vector, Ad.CMV*VLDLR*, contains the human very low density lipoprotein (VLDL) receptor (VLDLR) cDNA that mediates the binding and internalization of VLDL as well as other ligands [24, 25]. This construct was used to evaluate the ability of the Ad vector to modify cells with a gene coding for a protein expressed on the cell surface that is potentially involved in lipid transport in the placenta [26].

MATERIALS AND METHODS

Cell Preparation and Culture

Cytotrophoblast cells were prepared from human term placentas as previously described by Kliman et al. [27]. This method, which utilizes serial trypsin-DNase digestions followed by Percoll gradient centrifugation to purify cells, yields a highly enriched preparation of cytotrophoblasts. After isolation, these cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 25 mM glucose, 25 mM Hepes, and 50 μg/ml gentamicin and supplemented with 10% heat-inactivated fetal calf serum (FCS).

JEG-3 choriocarcinoma cells (American Type Culture Collection, Rockville, MD) and the b30 clone of BeWo cells (a gift from Dr. A.I. Schwartz, Washington University, St. Louis, MO) were maintained in the culture medium described above.

Recombinant Ad Vectors: Ad.CMVlacZ and Ad.CMVVLDLR

The production of the replication-deficient Ad vectors by homologous recombination techniques has been described in detail elsewhere [20, 21]. Briefly, the vectors were constructed from an adenovirus type 5 mutant that lacks most of the viral sequence regions E1a and E1b and a portion of E3. By homologous recombination techniques, either *E. coli LacZ* cDNA or the human VLDL receptor cDNA, driven by the human cytomegalovirus (CMV) promoter, was inserted into the viral genome.

Large-Scale Production, Purification, and Titration of Ad.CMVlacZ and Ad.CMVVLDLR

Ad stocks were propagated as previously described by Engelhardt et al. [23]. Briefly, human embryonic kidney 293 cells were grown to 90% confluency in 150-mm culture dishes containing DMEM supplemented with 10% FCS. At the time of infection with the recombinant Ad vectors (1×10^{10} viral particles per plate), the culture medium was replaced with DMEM containing 2% FCS. Thirty-six hours after infection, immediately before the cytopathic effect was complete, the cells were scraped and pelleted by centrifugation at 4000 × *g* for 20 min at 4°C. The cell pellet was freeze-thawed three times and subjected to centrifugation at 3000 × *g* for 10 min at 4°C. The supernatant, which contained the virus, was layered onto a discontinuous CsCl gradient and subjected to ultracentrifugation at 50 000 × *g* for 4 h at 4°C. The collected viral band was subjected once more to the same gradient centrifugation for 15 h at 4°C. The collected viral band was desalted in a Sephadex G25 column (Pharmacia, Piscataway, NJ). The viral concentration was determined by spectrophotometry (at 260 nm).

Plasmid Vector: pAd.CMVlacZ

The reporter gene sequence of β-gal was placed into a cassette in the intermediate plasmid vector, pAd.BglII, under the control of the CMV enhancer and promoter [28]. By this means, the promoter and reporter gene were flanked by the origin of replication and packaging sequence of the human type 5 Ad. Plasmid DNA amplification and purification were performed using standard techniques [29].

DiI-Labeling of VLDL

Human VLDL (d = 1.006–1.018 mg/ml) was labeled with 3',3'-dioctadecylindocarbocyanine percolate (DiI) through use of methods described by Xu et al. [30]. Briefly, 1.56 mg of VLDL protein was incubated in 3 ml of lipoprotein-deficient plasma containing 75 μl of DiI dissolved in dimethyl sulfoxide (3 mg DiI/ml) for 12 h at 37°C. The volume was then adjusted to 12 ml with saline; next, the density was adjusted to 1.02 g with KBr. DiI-labeled VLDL was re-isolated by ultracentrifugation at 49 000 × *g* at 4°C for 20 h. The diI-labeled VLDL fraction was removed and dialyzed against saline for 24 h at 4°C.

Gene Transfer into Choriocarcinoma Cells

Ad-mediated gene transfer into choriocarcinoma cells was evaluated by detection of vector-specific protein expression. To accomplish this, JEG-3 and BeWo cells were cultured in 3.5-cm^2 plastic dishes (Falcon, Becton Dickinson, Lincoln Park, NJ). At 50–60% confluency (approximately 2×10^6 cells), the cells were infected with 1×10^4, 2×10^4, or 4×10^4 viral particles per cell of either Ad.CMV*lacZ* or Ad.CMV*VLDLR*.

To detect expression of β-gal, 48 h after exposure to the recombinant Ad vectors, the cells were fixed and stained with the β-gal substrate, X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactosidase), as previously described [31]. With this technique, the presence of β-gal activity is indicated by a blue stain that is present in cells in which gene transfer and expression have been successful.

Immunohistochemistry was used to evaluate the ability of Ad-mediated gene transfer to elevate the levels of VLDLR expression. BeWo cells were cultured on 22 × 22-mm glass coverslips placed in 3.5-cm^2 culture dishes before being infected with 4×10^4 viral particles per cell of either Ad.CMVlacZ or Ad.CMV*VLDLR*. Forty-eight hours after infection, the cells were fixed with 2% paraformaldehyde and

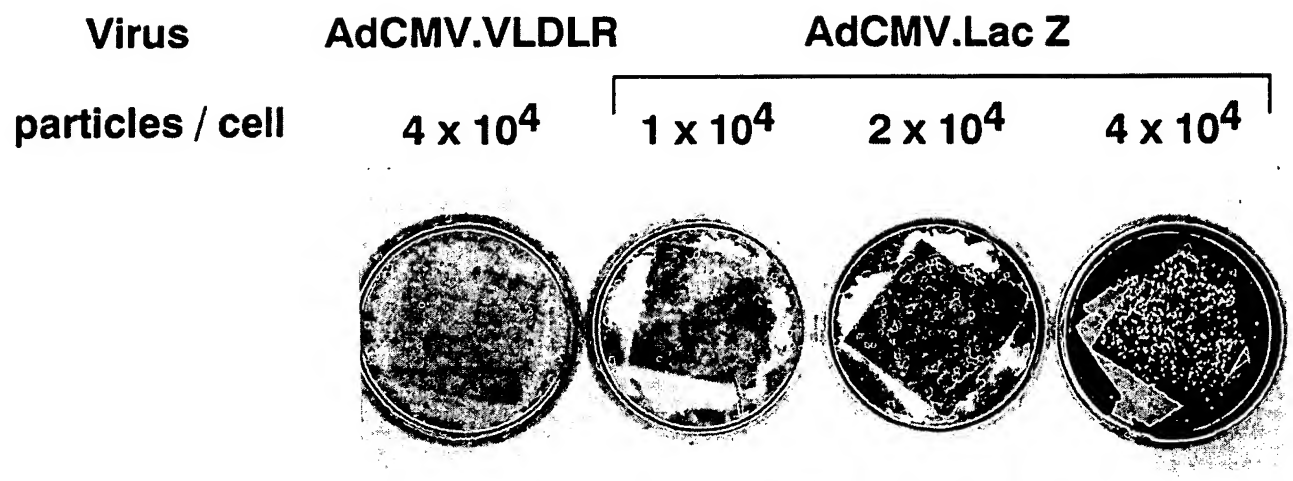


FIG. 1. Expression of β-gal in BeWo cells after transduction by either 4 × 10^4 particles per cell of Ad.CMV*VLDLR* or 1 × 10^4 particles per cell, 2 × 10^4 viral particles per cell, or 4 × 10^4 viral particles per cell of Ad.CMV*lacZ*.

0.2% glutaraldehyde in PBS for 30 min at room temperature. Immunohistochemistry was performed by means of rabbit antiserum directed against human VLDLR as previously described by Wittmaack et al. [26]. Sequential incubations were performed according to the method of Cartun and Pedersen [32] and included 10% normal horse serum for 30 min, primary antiserum at 37°C for 1 h, secondary biotinylated antibody at 37°C for 45 min, streptavidin-biotinylated horseradish peroxidase complex reagent at 37°C for 30 min, and three 5-min washes in PBS. The cells were then exposed to chromogen reaction solution (0.035% diaminobenzidine and 0.03% H_2O_2) for 10 min, washed in tap water for 5 min, counterstained in hematoxylin, dehydrated, cleared, and mounted.

To determine whether transduced BeWo cells were capable of binding and internalizing increased amounts of VLDL, cells that had been infected with 4 × 10^4 viral particles per cell of either Ad.CMV*lacZ* or Ad.CMV*VLDLR* for 48 h were incubated with 60 μg of DiI-labeled VLDL per dish as described by Xu et al. [30]. Briefly, the cells were washed three times with PBS before being incubated with DiI-labeled VLDL in DMEM containing 0.5% BSA for 60 min. After five further washes with PBS, the uptake of DiI-labeled VLDL by transduced BeWo cells was examined by fluorescent microscopy. To perform competitive uptake studies, the cells were incubated with 50 μg of unlabeled VLDL per dish for 5 min before addition of the DiI-labeled VLDL. Sixty minutes after addition of the DiI-labeled VLDL, the cells were washed and examined for VLDL uptake. Cells infected with Ad.CMV*lacZ* were used as controls.

To examine the correlation between the transduction efficiency of the Ad vectors and cellular differentiation, BeWo and JEG-3 cells were cultured in the presence or absence of 8-bromo-cAMP (1.5 mM). Cells were infected with 4 × 10^4 viral particles per cell of the Ad vectors at 0, 24, or 48 h after the addition of cAMP to the culture medium. Forty-eight hours after infection, the cells were fixed and stained with X-gal.

In order to determine whether the reduced transduction efficiency of the Ad vectors following cAMP-induced cellular differentiation was the result of a reduction in infectivity and/or a reduction in transcriptional/translational efficiency, BeWo cells were cultured in the presence or absence of 8-bromo-cAMP (1.5 mM) for 24 h prior to infection with 4 × 10^4 particles per cell of Ad.CMV*lacZ*. Immediately or 24 h after infection, the cells were washed three times with PBS and subjected to trypsin digestion for collection of cells and removal of cell surface-associated virus. The cells were then pelleted by centrifugation at 3000 × *g* for 10 min.

DNA was extracted from the cell pellets according to the method of Gross-Bellard et al. [33]. Briefly, the cell pellets were incubated with proteinase K (0.5 mg/ml) in 50 mM Tris-HCl (pH 8.0), 100 mM EDTA, 100 mM NaCl, 1% SDS for 16 h at 65°C. The DNA was subsequently extracted two times with equal volumes of buffered phenol/chloroform. DNA was precipitated with 2 volumes of ethanol, pelleted at 12 000 × *g* for 10 min, rinsed in 70% ethanol, and air dried. The DNA pellet was rehydrated in 100 μl of water and quantified by spectrophotometry. Aliquots of 40 μg of DNA were separated by electrophoresis on a 1% agarose gel and transferred by standard Southern blot procedures [29]. After transfer, the blot was probed with a 300-bp fragment of the plasmid pAd.CMV*lacZ* according to the methods described by MacCalman and Blaschuk [34].

To examine the transcriptional/translational efficiency of pAd.CMV*lacZ* with respect to cellular differentiation, BeWo and JEG-3 cells were cultured in the presence or absence of 8-bromo-cAMP (1.5 mM). At 0, 24, or 48 h after the addition of cAMP to the culture medium, cells were washed

twice with serum-free DMEM. The cells were then incubated in 1 ml of serum free-medium containing 2 μg of plasmid with 10 μg lipofectamine (Gibco/BRL, Grand Island, NY). After a 5-h incubation, 1 ml of culture medium containing 20% FCS was added to the cells. At this stage, cAMP was added back to the culture medium of cells that had been previously treated. Forty-eight hours after transfection, the cells were fixed and stained with X-gal.

Gene Transfer into Trophoblast Cells

Freshly isolated cytotrophoblast cells were seeded at a density of 2×10^6 cells per 2.5-cm^2 culture dish. After 12, 36, or 60 h of culture, the cells were infected with Ad.CMV*lacZ* at a concentration of 4×10^4 viral particles per cell. Forty-eight hours after infection with the Ad vector, the cells were fixed and stained with X-gal.

All experiments were repeated on at least two separate occasions with different recombinant Ad preparations.

RESULTS

Choriocarcinoma Cells Are Readily Transduced by Recombinant Ad Vectors

There was a dose-response relationship between the number of viral particles added per cell and the transduction of both BeWo cells (Fig. 1) and JEG-3 cells (data not shown). The number of stained cells, as well as the intensity of staining, was greatest in cells infected with 4×10^4 viral particles per cell. A cytopathic effect was not observed at any of the virus concentrations used in this study. X-gal staining was not observed in cells infected with 4×10^4 viral particles per cell of Ad.CMV*VLDLR*, demonstrating that endogenous β-gal activity makes no significant contribution to the X-gal staining.

To test the ability of the recombinant Ad vectors to deliver a functional gene that could be involved in placental nutrient transport, we examined VLDLR expression in transduced BeWo cells using immunohistochemistry. Using a specific polyclonal antipeptide antibody directed against human VLDLR, we observed modest peroxidase staining in BeWo cells transduced with Ad.CMV*lacZ*, reflecting endogenous VLDLR expression (Fig. 2). The staining was substantially more intense in BeWo cells transduced by Ad.CMV*VLDLR*.

To determine the ability of this exogenous gene product to bind and internalize VLDL, BeWo cells were incubated with DiI-labeled VLDL. Cells infected with Ad.CMV*VLDLR* were capable of accumulating increased amounts of DiI-labeled VLDL as determined by fluorescence microscopy (Fig. 3). The internalized DiI-labeled VLDL particles were

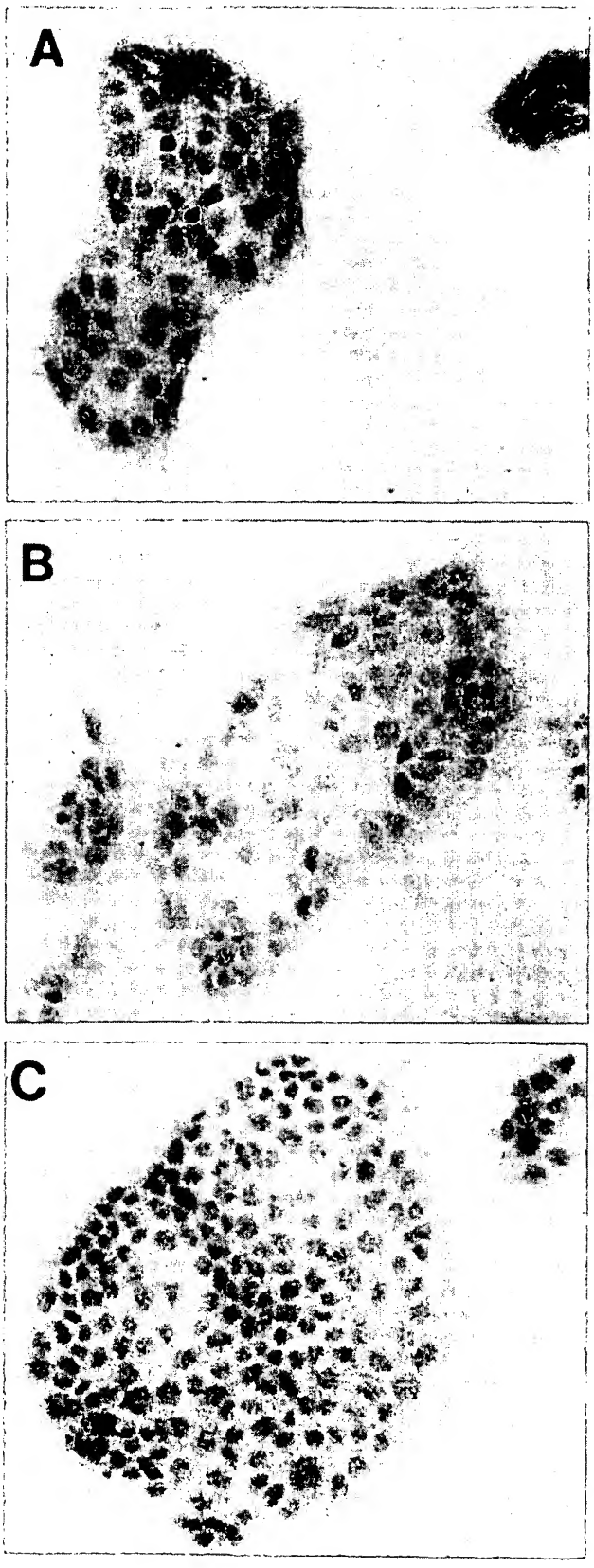


FIG. 2. Detection of VLDLR by immunohistochemistry in BeWo cells transduced by Ad.CMV*VLDLR* (A) or Ad.CMV*lacZ* (B) using an antiserum directed against human VLDLR. C) A negative control in which the antiserum was omitted.

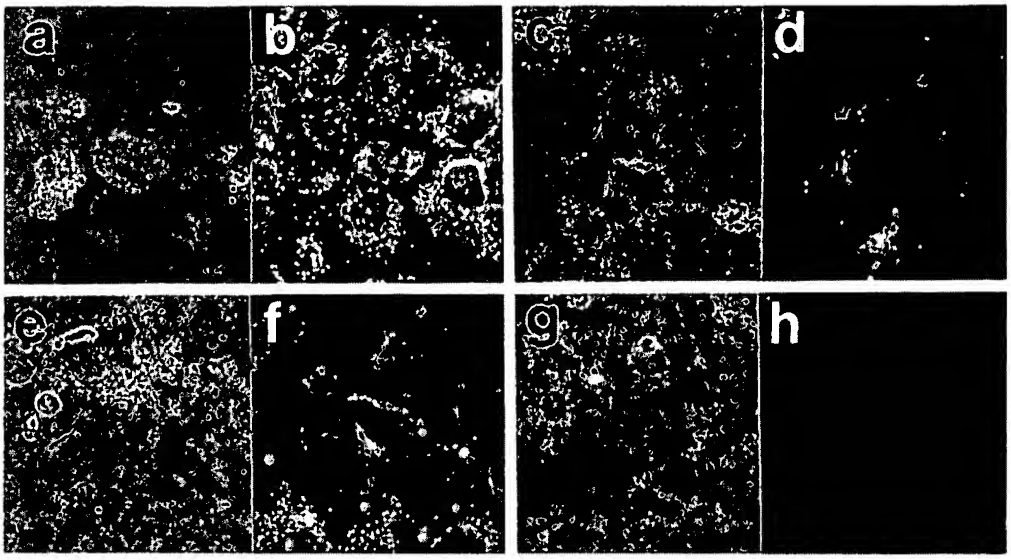


FIG. 3. BeWo cells were infected with 4×10^4 particles per cell of Ad.CMV*VLDLR* (a, b, c, d, g, h) or Ad.CMV*lacZ* (e, f). After 48 h the cells were incubated with DiI-labeled VLDL (60 μg/ml) (a, b, e, f) or DiI-labeled VLDL (60 μg/ml) with unlabeled VLDL (50 μg/ml) (c, d) or without added labeled VLDL (g, h). BeWo cells transduced with Ad.CMV*VLDLR* showed greater accumulation of DiI-labeled VLDL than in Ad.CMV*lacZ*-infected cells, and addition of unlabeled VLDL reduced uptake of the DiI-labeled lipoprotein. Phase contrast and corresponding fluorescent microscopy are shown.

observed to collect in endosomes in a punctate pattern. The uptake of labeled VLDL was diminished in the presence of unlabeled VLDL, indicating a saturable and specific uptake mechanism. Only modest levels of DiI-labeled VLDL were taken up by BeWo cells infected with Ad.CMV*lacZ*. The accumulation of DiI-labeled VLDL particles in these cells is probably caused by the binding of DiI-labeled VLDL to endogenous VLDLR and possibly low density lipoprotein receptors (LDLR). These findings demonstrate that the recombinant Ad vector can direct expression of a functional plasma membrane receptor potentially involved in placental nutrient uptake.

Differentiation-Dependent Transduction Efficiency of Trophoblast Cells

Freshly isolated cytotrophoblast cells were readily transduced by Ad.CMV*lacZ*, as determined by X-gal staining (Fig. 4). The transduction efficiency of freshly isolated cytotrophoblast cells was comparable to that observed with the two choriocarcinoma cell lines. However, over time in culture, the transduction efficiency was markedly reduced. The reduction in transduction efficiency occurred concomitantly with the terminal differentiation of the mononucleate cytotrophoblasts into multinucleated syncytial trophoblast cells.

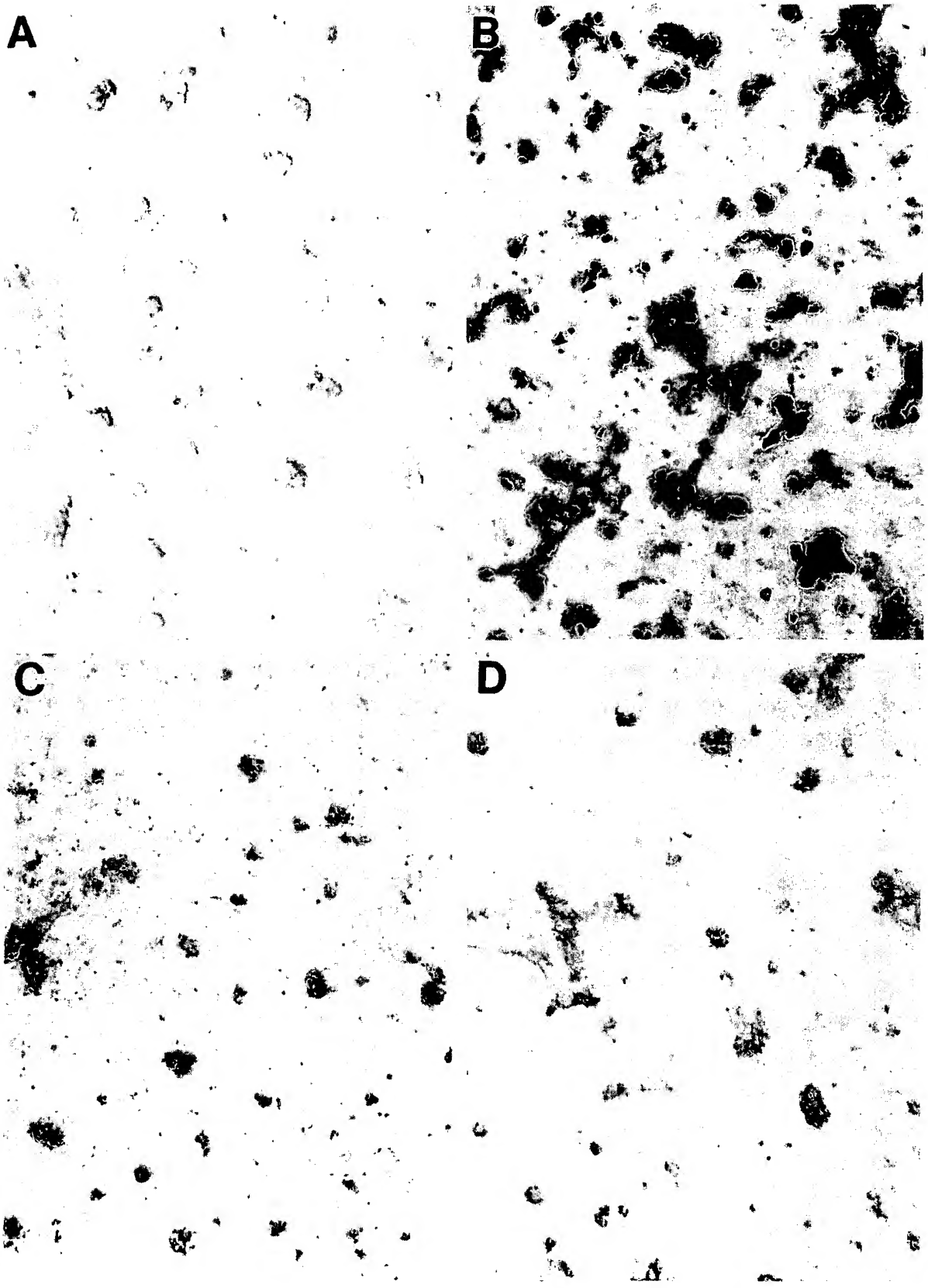
There was no change in the transduction efficiency of either BeWo or JEG-3 cells by Ad.CMV*lacZ* during 96 h of culture, as determined by the X-gal reaction (data not shown). The addition of 1.5 mM 8-bromo-cAMP to the culture medium induced BeWo cell fusion, which was initially observed after 24 h and was extensive after 48 h. As differentiation into syncytial structures progressed, there was a marked reduction in the number of cells that stained blue (Fig. 5). In contrast, the addition of the cAMP analog to JEG-3 cells had no effect on cell fusion and did not alter the transduction efficiency of these cells by Ad.CMV*lacZ* (Fig. 5).

Differentiation-Dependent Transduction Efficiency of Choriocarcinoma Cells Appears to Be Caused by a Reduction in Infectivity

Southern blot analysis, used to examine DNA samples prepared from BeWo cells transduced with Ad.CMV*lacZ* in the presence or absence of cAMP, demonstrated that a single band of 3.5 kb, corresponding to the *LacZ* gene, was present in all the samples analyzed (Fig. 6). Increased levels of the *LacZ* gene were detected in BeWo cells infected with Ad.CMV*lacZ* in the absence of cAMP for 24 h. In contrast, cells cultured in the presence of cAMP did not exhibit increased levels of the *LacZ* gene after exposure to the Ad vector for the same amount of time.

Although treatment with 8-bromo-cAMP markedly diminished BeWo cell transduction by Ad.CMV*lacZ*, the cAMP analog did not alter transfection efficiency of either BeWo or JEG-3 cells with the plasmid pAd.CMV*lacZ*, in which the

FIG. 4. Expression of β-gal in isolated human cytotrophoblasts after infection with 4×10^4 particles per cell of either Ad.CMV*VLDLR* (A) or Ad.CMV*lacZ* (B-D). Cells were cultured for 12 h (A and B), 36 h (C), or 60 h (D) before infection with the Ad vectors. Forty-eight hours after infection, the cells were fixed and stained with X-gal.



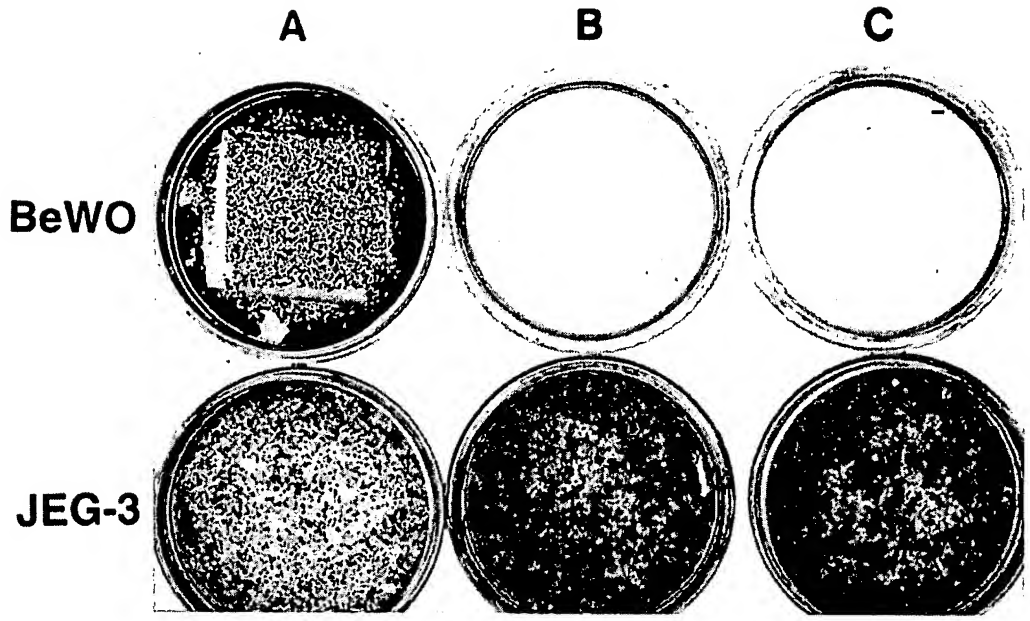


FIG. 5. Expression of β-gal in BeWo cells (upper row) and JEG-3 cells (lower row) after transduction by Ad.CMV *lacZ* (4×10^4 particles per cell). Cells were cultured in the presence of 8-bromo-cAMP (1.5 mM) for 0 h (column A), 24 h (column B), or 48 h (column C) before infection with the Ad vector. Forty-eight hours after infection, the cells were fixed and stained with X-gal.

LacZ gene is under the control of the same CMV promoter used in the recombinant Ad vectors (Fig. 7).

DISCUSSION

Recombinant Ad have been used to transfer genes into terminally differentiated cells of adult tissues, including those of the central nervous system. Our findings demonstrate that it is possible to use a replication-deficient Ad vector to transfer and express exogenous genes in cytotrophoblast cells and choriocarcinoma cells in vitro. However, the transduction efficiency of these trophoblast cells by recombinant Ad vectors is dramatically reduced as the cells undergo terminal differentiation. Thus, there must be cell lineage-specific factors that regulate susceptibility to Ad-mediated transduction. These findings are consistent with the recently described differential efficiency of Ad-mediated gene transfer into skeletal muscle cells of different maturity [12, 13]. In these studies, recombinant Ad vectors were capable of efficiently transducing myoblasts but not myocytes.

The transduction efficiency of the human Ad is dependent on its infectivity for the target cell and the transcriptional/translational efficiency of the exogenous gene. In order to define the factor(s) that may be responsible for the reduced transduction efficiency of the recombinant Ad in differentiated trophoblast cells, we transfected BeWo and JEG-3 cells with an Ad plasmid containing the *LacZ* gene and the same CMV promoter used to drive gene expression in the recombinant Ad vectors. As determined by the X-gal reaction, the transfection efficiency of this plasmid was not

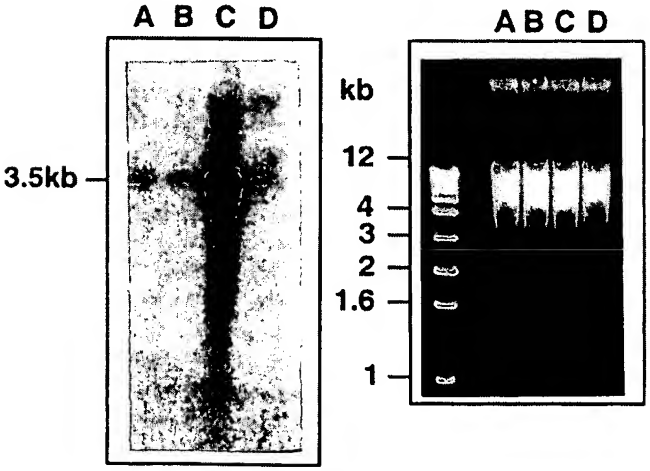
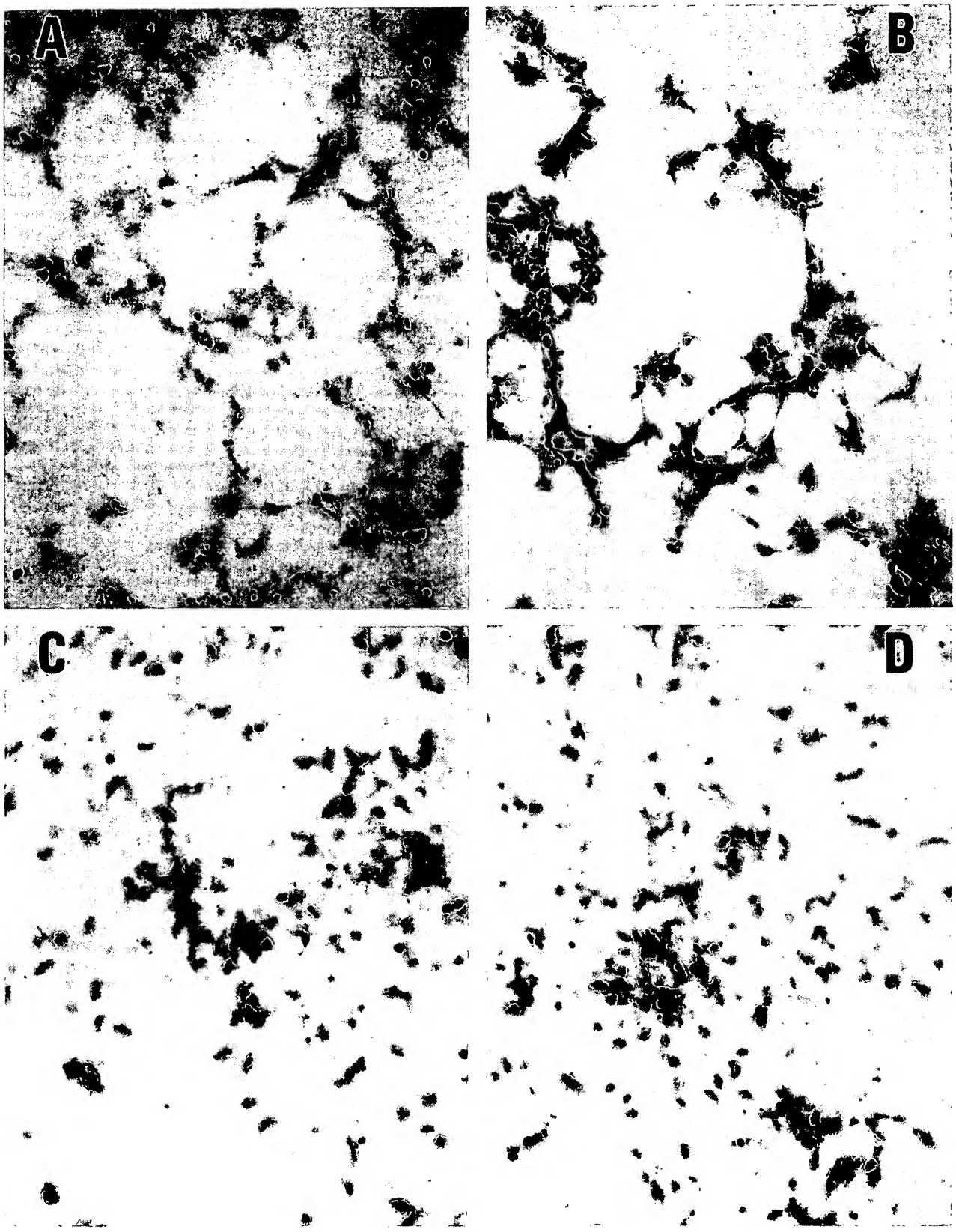


FIG. 6. Left) Autoradiogram of a Southern blot containing aliquots (40 μg/lane) of DNA extracted from BeWo cells cultured for 24 h in the absence (lanes A and C) or presence (lanes B and D) of 8-bromo-cAMP (1.5 mM) prior to exposure to 4×10^4 particles per cell of Ad.CMV*lacZ*. Cells were harvested immediately (lanes A and B) or 24 h (lanes C and D) after exposure to the Ad vector. The blot was probed with a cDNA fragment prepared from pAd.CMV*lacZ*. Right) Photograph of the ethidium bromide-stained gel prior to Southern transfer.

FIG. 7. Expression of β-gal in BeWo cells (A and B) and JEG-3 cells (C and D) after transfection by pAd.CMV*lac Z*. Cells were cultured in the presence of 8-bromo-cAMP (1.5 mM) for 0 h (A and C) or 48 h (B and D) before transfection with pAd.CMV*lacZ*. Forty-eight h after transfection, the cells were fixed and stained with X-gal.



affected by cellular differentiation. To detect changes in the infectivity of the Ad vectors after cellular differentiation, we used Southern blot analysis to determine the number of copies of the *LacZ* gene in cells that had been transduced by Ad.CMV*lacZ*. As expected, the number of gene copies was high in cells not treated with cAMP that are readily transduced by the Ad vectors. However, the number of copies of the *LacZ* gene did not increase in BeWo cells that had undergone cAMP-induced differentiation. These results suggest that the reduced transduction efficiency of the Ad vectors following cellular differentiation is caused, at least in part, by a diminished infectivity. Taken together, the results suggest that trophoblasts cells lose the ability to internalize viral particles but are still capable of processing exogenous genetic material after terminal differentiation.

Entry of the human Ad into host cells is believed to involve the interaction of virus particles with two distinct receptors [35]. The initial binding event is mediated by a cell surface receptor that is as yet unidentified, while subsequent internalization/penetration of the virus is thought to be mediated by the interaction of the viral coat with alpha-v and beta-5 integrin subunits. Although these interactions have been documented for blood cells [35], skeletal muscle [13], and some epithelial cell lines [36, 37], it is not clear whether or not these events occur during Ad infection of other tissues. Recent studies have demonstrated that the alpha-v and beta-5 integrin subunits are expressed in both cytotrophoblast cells and syncytial trophoblasts in vitro [38, 39]. In addition, the infectivity of ovarian and mesothelial cancer cell lines does not appear to be dependent on the expression of alpha-v integrin subunits [19]. Taken together, these observations suggest that the internalization of Ad particles is complex and that it is likely that a change in expression of the alpha-v integrin subunit is not the cause of the reduced transduction efficiency observed in both cAMP-treated BeWo cells and normal syncytial trophoblasts in vitro. Although entry of the Ad vectors into the trophoblast cells may be the key process controlling transduction, it is not possible for us to determine whether or not alterations in other intracellular mechanisms involved in the processing of viral particles [40], such as the stepwise disassembly of viral proteins within endosomes or the transport of the genetic material to the nucleus, also contribute to the reduced transduction efficiency of the Ad vectors in differentiating trophoblasts.

The syncytial trophoblast of the placenta serves as the first line of defense of the fetus against maternal viremia. The infectibility of human and animal placental cells by Ad has been examined by others. Freund et al. [41] reported that type 2 Ad can infect dispersed human placental cells in culture. However, the type of cell infected (i.e., cytotrophoblast cells, syncytial trophoblasts, or nontrophoblast cells) was not determined in that study. In contrast, Rosztoczy et al. [42] and Sweet et al. [43] found that neither human nor guinea pig placental explants were susceptible to Ad infection. These latter reports are consistent with our findings that differentiated trophoblast cells are not transducible by recombinant Ad. Recent studies have demonstrated that the human trophoblast is not permissive to infection by a number of other viruses in vitro and in vivo. For example, cytotrophoblast cells and syncytial trophoblasts exhibit restricted permissiveness for human immunodeficiency virus type 1 [44, 45] and human CMV [46]. In addition, vaccinia virus and herpes simplex virus replication is slow and to low titers in trophoblast cells compared to placental fibroblasts and malignant transformed trophoblast cells [47, 48].

The mechanism(s) by which the trophoblast cells regulate viral infection remains poorly characterized. However, there is some evidence to indicate that low levels of viral receptor proteins expressed on the surface of trophoblast cells may account for some of these observations [44, 49].

Our results suggest that maternally administered recombinant Ad will not be useful for gene therapy of the placenta and fetus. Indeed, in preliminary experiments performed in pregnant mice and rats, we have found negligible expression of *LacZ* in the placenta after intravenous or intrauterine arterial injection of Ad.CMV*lacZ* in late pregnancy. However, *LacZ* expression was detected in the maternal liver in these experiments.

Ad infection of the fetus and neonate is a rare and generally fatal condition [50]. Although several mechanisms of infection have been proposed, the results of this study indicate that the syncytial trophoblast of the placenta presents an effective barrier against Ad infection in utero. Overriding this barrier by injecting Ad into the amniotic fluid of rodents and sheep has been recently shown to cause epithelial hyperplasia and squamous metaplasia and to trigger a dramatic immune response in the fetus [51].

In summary, we have demonstrated that the syncytial trophoblast of the human placenta is resistant to Ad-mediated gene transfer in vitro. However, cytotrophoblast cells and presumably the trophectoderm of the blastocyst are susceptible to Ad-mediated transduction. That differentiated trophoblast cells are relatively resistant to Ad-mediated gene transfer while transfection efficiency with plasmids is retained suggests that differentiation affects Ad infectivity. Hence, our results provide insight into the protective barrier provided by the trophoblast layer against viral infection. They also suggest useful systems (i.e., BeWo cells and differentiating cytotrophoblasts) with which acquired resistance to Ad transduction/infection can be explored.

REFERENCES

1. Evain-Brion D, Alsat E. Epidermal growth-factor receptor and human fetoplacental development. J Pediatr Endocrinol 1994; 7:295–302.
2. Aldoretti FW, Hay WW. Fetal nutrition. Nutr Res 1995; 14:929–965.
3. Landor M. Maternal-fetal transfer of immunoglobulins. Ann All Asthma Immunol 1995; 74:279–283.

4. Boyd JD, Hamilton WJ, Milton WJ. The Human Placenta. London: Macmillan; 1980.
5. Cunningham FG, MacDonald PC, Grant NF. The placenta and fetal membranes. In: Williams Obstetrics, 18 ed. Norwalk, CT: Appelton and Lang; 1989: 39.
6. Aplin JD. Implantation, trophoblast differentiation and haemochorial placentation: mechanistic evidence *in vivo* and *in vitro*. J Cell Sci 1991; 99:681–692.
7. Salafia CM, Vogel CA, Vintzileos AM, Bantham KF, Pezzullo J, Silberman L. Placental pathologic findings in preterm birth. Am J Obstet Gynecol 1991; 165:934–938.
8. Zhou Y, Damsky CH, Chiu K, Roberts JM, Fisher SJ. Preeclampsia is associated with abnormal expression of adhesion molecules by invasive cytotrophoblasts. J Clin Invest 1993; 91:950–960.
9. Redline R, Patterson R. Preeclampsia is associated with an excess of immature intermediate trophoblast. Lab Invest 1995; 70:pA 148.
10. Barker DJP. Outcome of low birthweight. Horm Res 1994; 42:223–230.
11. Redline RW. Placental pathology: a neglected link between basic disease mechanisms and untoward pregnancy outcome. Curr Opin Obstet Gynecol 1995; 7:10–15.
12. Acsadi G, Jiao S, Jani A, Duke D, Williams P, Chong W, Wolff JA. Direct gene transfer and expression into rat heart *in vivo*. New Biol 1991; 3:71–81.
13. Acsadi G, Jani G, Massie B, Simoneau M, Holland P, Blaschuk K, Karpati G. A differential efficiency of adenovirus-mediated *in vivo* gene transfer into skeletal muscle cells of different maturity. Hum Mol Genet 1994; 3:579–584.
14. Davidson BL, Allen ED, Kozarsky KF, Wilson JM, Roessler J. A model system for in vivo gene transfer into the central nervous system using an adenoviral vector. Nat Genet 1993; 3:219–223.
15. Le Gal La Salle J, Robert JJ, Berrard S, Ridoux V, Stratford-Perricaudet LD, Perricaudet M, Mallet J. An adenovirus vector for gene transfer into neurons and glia in the brain. Science 1993; 259:988–990.
16. Chang MW, Ohno T, Gordon D, Lu MM, Nabel GJ, Nabel EG, Leiden JM. Adenovirus-mediated transfer of the herpes simplex virus thymidine kinase gene inhibits vascular smooth muscle cell proliferation and neointima formation following balloon angioplasty of the rat carotid artery. Mol Med 1995; 1:172–181.
17. Clayman GL, El-Naggar AK, Roth JA, Zhang WW, Goepfert H, Taylor DL, Liu TJ. *In vivo* molecular therapy with p53 adenovirus for microscopic residual head and neck squamous carcinoma. Cancer Res 1995; 55:1–6.
18. Korst RJ, Bewig B, Crystal RG. *In vitro* and *in vivo* transfer and expression of human surfactant SP-A- and SP-B-associated protein cDNAs mediated by replication-deficient, recombinant adenoviral vectors. Hum Gene Ther 1995; 6:277–287.
19. Smythe WR, Hwang HC, Elshami AA, Amin KM, Albelda SM, Kaiser LR. Differential sensitivity of thoracic malignant-tumors to adenovirus-mediated drug sensitization gene therapy. J Thorac Cardiovasc Surg 1995; 109:626–631.
20. Berkner KL. Development of adenovirus vectors for the expression of heterologous genes. Biotechniques 1988; 6:616–629.
21. Berkner KL. Expression of heterologous sequences in adenoviral vectors. Curr Top Microbiol Immunol 1992; 158:39–66.
22. Kozarsky KF, Wilson JM. Gene therapy: adenovirus vectors. Curr Opin Genet Dev 1993; 3:499–503.
23. Engelhardt JF, Yang Y, Stratford-Perricaudet LD, Allen ED, Kozarsky K, Perricaudet M, Yankaskas JR, Wilson JM. Direct gene transfer of human CFTR into human bronchial epithelia of xenografts with E1-deleted adenoviruses. Nat Genet 1993; 4:27–33.
24. Gavfels ME, Caird M, Britt D, Jackson CL, Patterson D, Strauss III JF. Cloning of a cDNA encoding a putative human very low density lipoprotein/apolipoprotein E receptor and assignment of the gene to chromosome 9pter-p23. Somatic Cell Mol Genet; 1994; 19:557–569.
25. Battey FD, Gavfels ME, Fitzgerald DJ, Argraves WS, Chappell DA, Strauss III JF, Strickland DK. The 39-kDa receptor-associated protein regulates ligand binding by the very low density lipoprotein receptor. J Biol Chem 1994; 269:23268–23273.
26. Wittmaack FM, Gafvels ME, Bronner M, Matsuo H, McCrae KR, Tomaszewski JE, Robinson SL, Strickland DK, Strauss III JF. Localization and regulation of the human very low density lipoprotein/apolipoprotein-E receptor: trophoblast expression predicts a role for the receptor in placental lipid transport. Endocrinology 1995; 136:340–348.
27. Kliman HJ, Nestler JE, Sermasi E, Sanger JM, Strauss III JF. Differential transduction of cytotrophoblasts isolated from human term placentae. Endocrinology 1986; 118:1567–1582.
28. Kozarsky KF, McKinley DR, Austin LL, Raper SE, Stratford-Perricaudet LD, Wilson JM. *In vivo* correction of low density lipoprotein receptor deficiency in the Watnabe heritable hyperlipidemic rabbit with recombinant adenoviruses. J Biol Chem 1994; 269:13695–13702.
29. Davis LG, Dibner MD, Battey JF. Basic Methods in Molecular Biology. New York: Elsevier Science Publishing Co. Ltd.; 1986.
30. Xu Q, Buhler E, Steinmetz A, Schonitzer D, Bock G, Jurgens G, Wick G. A high-density-lipoprotein receptor appears to mediate the transfer of essential fatty acids from high-density lipoprotein to lymphocytes. Biochem J 1992; 287:395–401.
31. Forsayeth JR, Garcia PD. Adenovirus-mediated transfection of cultured cells. Biotechniques 1994; 17:354–359.
32. Cartun RW, Pedersen CA. An immunocytochemical technique offering increased sensitivity and lowered cost with streptavidin-horseradish peroxidase conjugate. J Histotechnol 1989; 12:273–280.
33. Gross-Bellard M, Oudet P, Chambon P. Isolation of high molecular weight DNA from mammalian cells. Eur J Biochem 1973; 36:32–38.
34. MacCalman CD, Blaschuk OW. Gonadal steroids regulate N-cadherin mRNA levels in the mouse testis. Endocrine 1994; 2:157–163.
35. Huang SA, Endo RI, Nemerow GR. Up-regulation of integrins alpha-v-beta-3 and alpha-v-beta-5 of human monocytes and T-lymphocytes facilitates adenovirus-mediated gene delivery. J Virol 1995; 69:2257–2263.
36. Wickham TJ, Filardo EJ, Cheresh DA, Nemerow GR. Integrin alpha-v-beta-5 selectively promotes adenovirus-mediated cell-membrane permeabilization. J Cell Biol 1994; 127:257–264.
37. Goldman MJ, Wilson JM. Expression of $\alpha_v\beta_5$ integrin is necessary for efficient adenovirus-mediated gene transfer in human airway. J Virol 1995; 69:5951–5958.
38. Omigbodun A, Tessler C, Coukos G, Hoyer J, Coutifaris C. Human trophoblast adhesion to osteopontin is regulated by cell differentiation and divalent cations. J Soc Gynecol Invest 1995; 2: Abstract 199.
39. Campbell S, Swann HR, Seif MW, Kimber SJ, Aplin JD. Cell adhesion molecules on the oocyte and preimplantation human embryo. Mol Hum Reprod 1995; 1:1571–1578.
40. Greber UF, Willets M, Webster P, Helenius A. Step-wise dismantling of adenovirus 2 during entry into the cells. Cell 1993; 75:477–486.
41. Freund E, Eller M, Kellner K, McGuire PM. Evidence that adenovirus type 2 can infect human placenta *in vitro*. Placenta 1981; 2:265–270.
42. Rosztoczy I, Sweet C, Toms GL, Smith H. Replication of influenza virus in organ cultures of human and simian urogenital tissues and human foetal tissues. Br J Exp Pathol 1975; 56:322–330.
43. Sweet C, Collie MH, Toms GL, Smith H. The pregnant guinea-pig as a model for studying influenza virus infection in utero: infection of foetal tissues in organ culture and *in vivo*. Br J Exp Pathol 1977; 58:133–139.
44. Douglas GC, Fry GN, Thirkill T, Holmes E, Hakim H, Jennings M, King BF. Cell-mediated infection of human placental trophoblast with HIV *in vitro*. Aids Res Hum Retroviruses 1991; 7:735–740.
45. McGann KA, Collman R, Kolson DL, Gonzalez-Scarano F, Coukos G, Coutifaris C, Strauss III JF, Nathanson N. Human immunodeficiency virus type 1 causes productive infection of macrophages in primary placental cell cultures. J Infect Dis 1994; 169:746–753.
46. Toth FD, Mosborgpetersen P, Kiss J, Aboagyemathiesen G, Hager H, Juhl CB, Gergely L, Zdravkovic M, Aranyosi J, Lampe L, Ebbesen P. Interactions between human-immunodeficiency-virus-type-1 and human cytomegalovirus in human term syncytiotrophoblast cells coinfected with both viruses. J Virol 1995; 69:2223–2232.
47. Norskov-Lauritsen N, Aboagye-Mathiesen G, Juhl CB, Petersen PM, Zachar V, Ebbesen P. Herpes simplex virus infection of cultured human term trophoblast. J Med Virol 1992; 36:162–166.
48. Norskov-Lauritsen N, Zachar V, Petersen PM, Hager H, Aboagye-Mathiesen G, Ebbesen P. *In vitro* infection of human placental trophoblast by wild-type vaccinia virus and recombinant virus expressing HIV envelope glycoprotein. Res Virol 1992; 143:321–328.
49. David FJE, Tran HC, Serpente N, Autran B, Vaquero C, Dijan V, Menu E, Barre-Sinoussi F, Chaquat G. HIV infection of choriocarcinoma cell lines derived from human placenta: the role of membrane CD4 and Fc-Rs into HIV entry. Virology 1995; 208:784–788.
50. Montone KT, Furth EE, Pietra GG, Gupta PK. Neonatal adenovirus infection—a case report with in-situ hybridization confirmation of ascending intrauterine infection. Diagn Cytopathol 1995; 12:341–344.
51. McCray PB, Armstrong K, Zabner J, Miller DW, Koretzky GA, Couture L, Robillard JE, Smith AE, Welsh MJ. Adenoviral-mediated-gene-transfer to fetal pulmonary epithelia *in vitro* and *in vivo*. J Clin Invest 1995; 95:2620–2632.